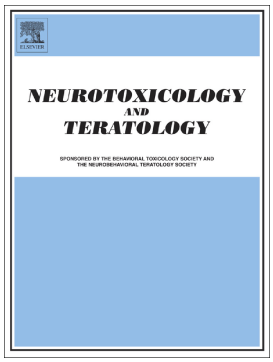

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In vitro protective activity of South Australian marine sponge and macroalgae extracts against amyloid beta ($A\beta_{1-42}$) induced neurotoxicity in PC-12 cells

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Abstract

South Australia is a biodiversity hotspot of marine sponges and macroalgae. This study aimed to evaluate the potential neuroprotective activity of extracts from these two marine sources by reducing the toxicity of human amyloid beta $A\beta_{1-42}$ in a cell model assay using PC-12 cells. A total of 92 extracts (43, 13, 16, and 20 extracts from sponge of 8 orders and 17 families, green algae of 3 orders and 4 families, brown algae of 6 orders and 8 families, and red algae of 5 orders and 10 families, respectively) were initially screened at three different concentrations (0.25, 2.5 and 25 µg/ml) to evaluate their toxicity using the MTT assay. About half of these extracts (26, 6, 5, and 10 extracts from sponge, green algae, brown algae, and red algae, respectively) showed some cytotoxicity, and were hence excluded from further assays. The rest of extracts (45 extracts in total) at 0.25 and 25 µg/ml were subsequently screened in a neuroprotection assay against $A\beta_{1-42}$ cytotoxicity. A cell viability reduction of 30% was observed in the MTT assay when the cells were treated with 1µM $A\beta_{1-42.}$ 29 extracts (13, 4, 7, and 5 extracts from sponge, green algae, brown algae, and red algae, respectively) reduced the toxicity induced by $A\beta_{1-42}$ ($P<0.05$), indicating neuroprotective activity. These results demonstrate that marine sponge and macroalgae form a broad spectrum are promising sources of

neuroprotective compounds against the hallmark neurotoxic protein in Alzheimer's disease (AD).

Keywords: Marine sponges; macroalgae; neuroprotective activity; amyloid beta; South Australia

1- Introduction:

Alzheimer's disease (AD) is a neurodegenerative disease responsible for 60-80% of dementia cases (Alzheimer'sAssociation, 2014). Current treatment strategies for AD mostly target acetylcholinesterase and the N-methyl-D-aspartate (NMDA) receptor. However, these treatments can only mitigate some of the cognitive and memory loss symptoms and are not considered disease-modifying. Hence, the development of new treatments for AD are required (Scarpini et al., 2003).

One of the main hallmarks of AD is the presence of amyloid beta (Aβ) protein that forms plaques in the brain. $A\beta_{1-40}$ and $A\beta_{1-42}$ are major forms generated from the cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase (Hussain et al., 1999). It is suggested that the aggregation and diminished clearance are pathogenic factors of AD (Hardy and Selkoe, 2002). Animal studies demonstrate that amyloid plaques are correlated with memory defects (Hsiao et al., 1996). For that reason, targeting Aβ may be considered an effective approach in the treatment of AD (Hardy and Selkoe, 2002).

Marine sponges, one of the oldest multicellular animals on the planet (Hentschel et al., 2002), are a rich source of natural compounds contributing more than 30% of all compounds discovered from marine organisms (Mehbub et al., 2014). These compounds possess a spectra of biological activities including anti-viral, anti-bacterial, and anti-inflammatory properties (Mayer et al., 2013). A recent review of

neuroprotective compounds from marine sponges ascribed a variety of mechanisms to their neuroprotection, including glutamate and serotoninergic receptor activity, kinase inhibition, neuritogenic and anti-oxidant activity (Alghazwi et al., 2016a). Interestingly, seven out of 90 neuroprotective compounds were reported as sourced from Australian species.

Macroalgae (or seaweeds) have been known for their uses in food and as potential drug sources. Macroalgae can be classified based on the pigment colours into different phyla such as Chlorophyta, Ochrophyta (class Phaeophyceae), and Rhodophyta which are commonly named as the green, brown and red algae, respectively (Lobban and Harrison, 1994, Guiry, 2012). Macroalgae present a range of biological activities such as anti-viral, anti-bacterial, antioxidant, anti-cancer and neuroprotective activity (Wang et al., 2008, Lima-Filho et al., 2002, Kang et al., 2003, Kang et al., 2004, Aisa et al., 2005, Pangestuti and Kim, 2011). A recent review reported a total of 99 compounds isolated from macroalgae demonstrating neuroprotective activities (Alghazwi et al., 2016b). The mechanisms ascribed to these effects included inhibiting Aβ aggregation and acetylcholinesterase inhibition, decreasing oxidative stress and kinase activity, enhancing neurite outgrowth, anti-inflammatory activity and protecting dopaminergic neurons.

South Australian waters have more than 1000 different species of sponges that belong to 200 genera (Bergquist and Skinner, 1982). South Australia hosts one of the highest diversity of macroalgae, as it is home to over 1200 species with 62% of them as endemic (Phillips, 2001, Womersley, 1996). Few studies have reported neuroprotective activities of sponges and macroalgae collected in Australian waters,

with only seven neuroprotective compounds from sponges. Esmodil was shown to inhibit acetylcholinesterase (Capon et al., 2004), while debromohymenialdisine inhibited CDK5/p25, CK1, and GSK-3β (Zhang et al., 2012c). Four compounds (Lamellarins O1, Ianthellidone F, lamellarins O2 and O) were shown to inhibit β-site amyloid precursor protein cleaving enzyme (BACE) (Zhang et al., 2012a), in addition to Dictyodendrin J (Zhang et al., 2012b). Moreover, only 3 compounds isolated from macroalgae collected in Australia were shown to have demonstrated neuroprotective activity. Spiralisone A, spiralisone B, and chromone 6 showed inhibition of CDK5/p25, CK1δ and GSK3β kinases (Zhang et al., 2012d). Therefore the present study was conducted to evaluate the potential of South Australia marine sponge and macroalgae extracts as a source of neuroprotective compounds, with a focus on reducing the cytotoxicity of Aβ in neuronal PC-12 cells.

2- Materials and Methods

2.1- Samples collection

The Australian Institute of Marine Science (AIMS) provided all the samples used in this study. These samples were collected by hand whilst scuba diving or from shallows at low tide. They were frozen after a representative taxonomy sample was taken. All samples were collected in South Australia. The details of collections sites can be found in the Table 1.

The taxonomy information was provided by AIMS. Phylogenetic trees of these samples were conducted according to their class, order, family, genus, and species with a guide from http://www.algaebase.org/ (for algae samples) (Guiry and Guiry, 2014) and http://www.marinespecies.org/porifera/ (for sponge samples) (Van Soest et al., 2017).

The marine samples were divided in four different categories based on their class for sponges (Demospongiae) or phyla for macroalgae (Chlorophyta, Ochrophyta, and Rhodophyceae). For each category a separate phylogenetic distribution was constructed to distribute the class into order, family, genus, and species, respectively.

All the sponge samples were from Demospongiae class with a broad distribution of 8 orders and 17 families (Figure 1). In Chlorophyta, there were 3 orders and 4 families (Figure 2). In Phaeophyceae, there were 6 orders and 8 families (Figure 3). In Rhodophyceae, there were 5 orders and 10 families (Figure 4).

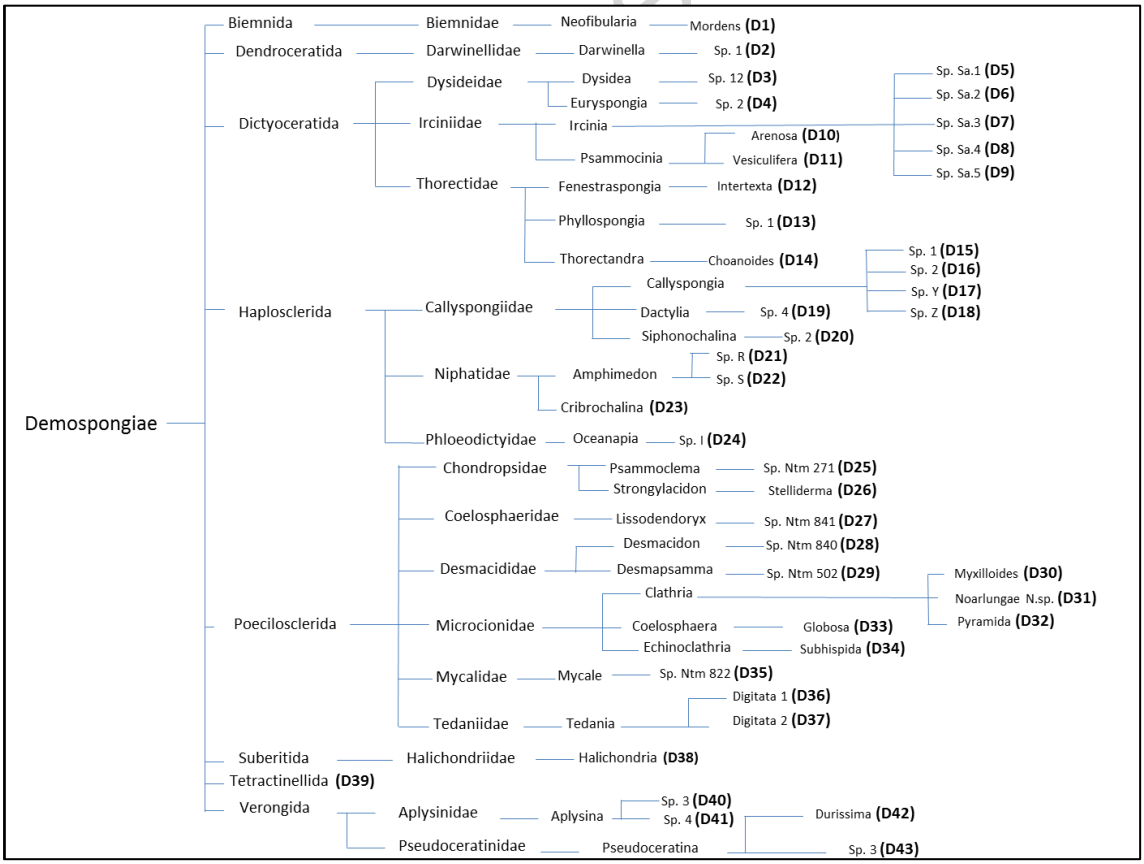

Figure 1: Phylogenetic distribution of sponge species among order, family, and genus from South Australia used in this study

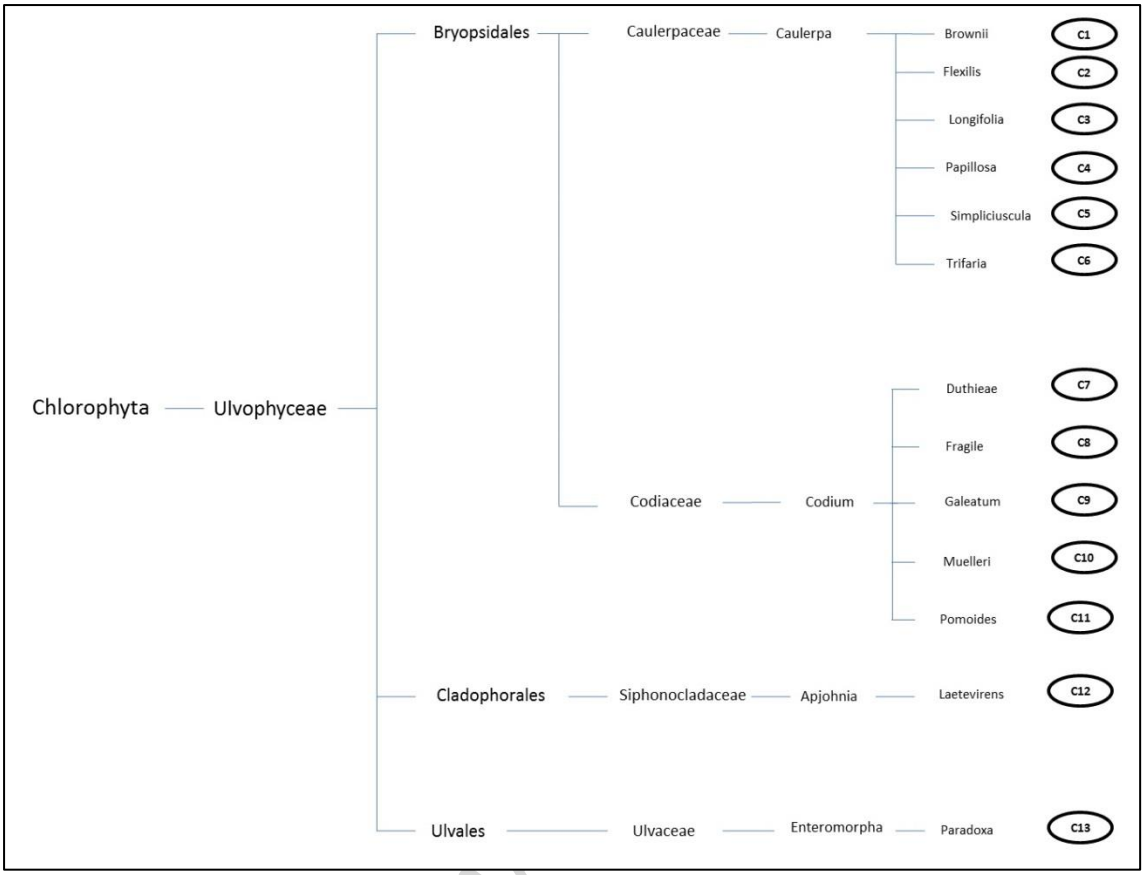

Figure 2: Phylogenetic distribution of green algae species among order, family, and genus from South Australia used in this study

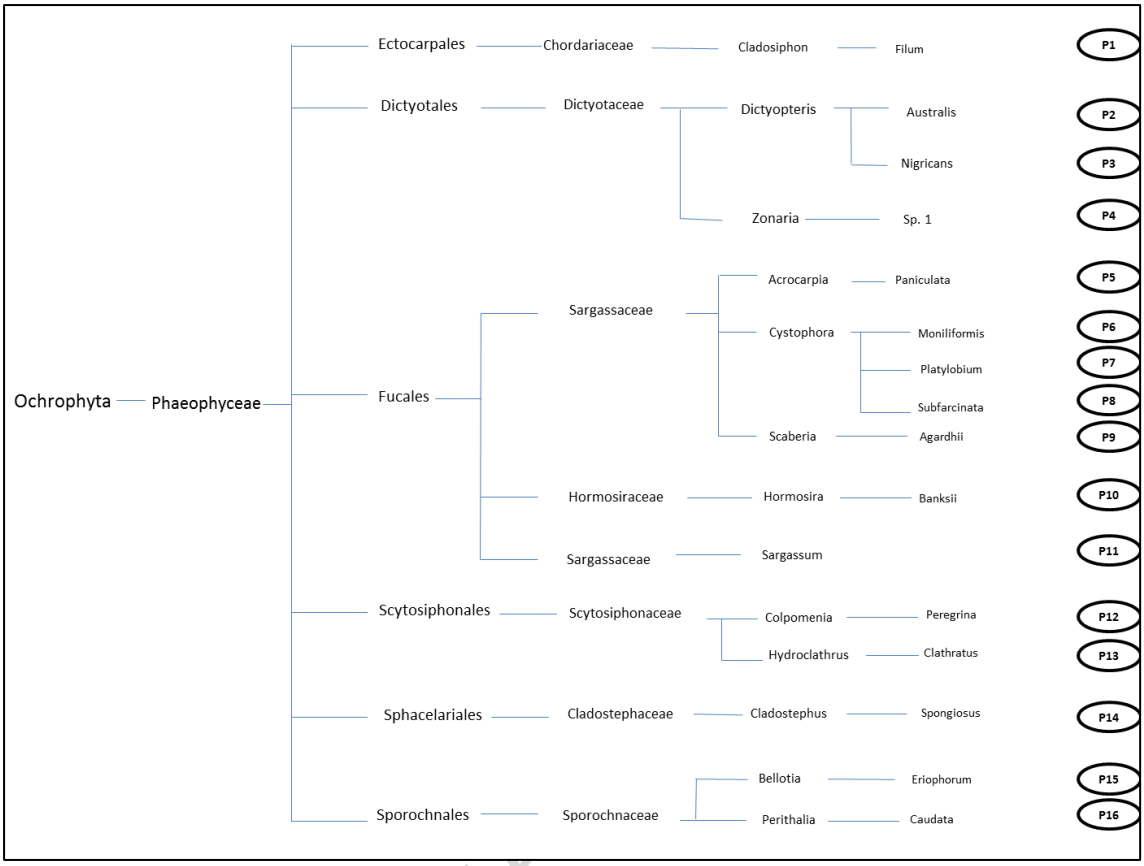

Figure 3: Phylogenetic distribution of brown algae species among order, family, and genus from South Australia used in this study

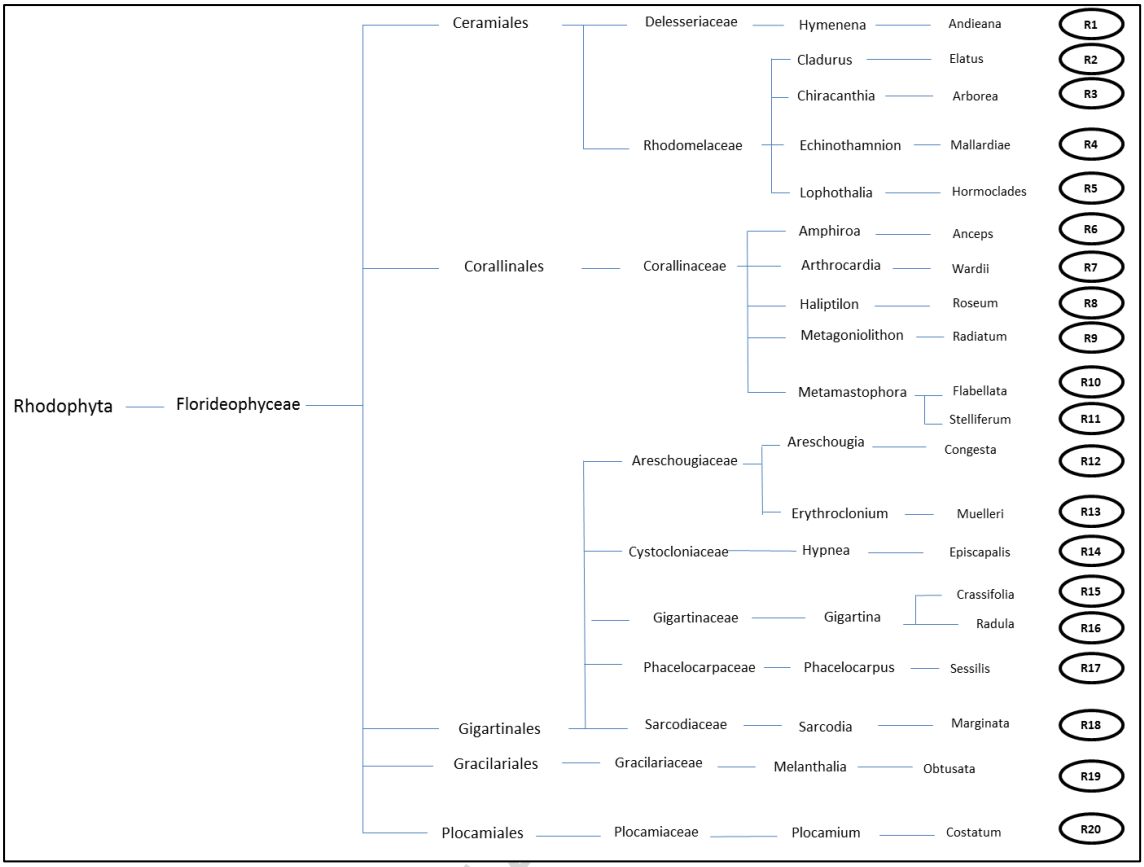

Figure 4: Phylogenetic distribution of red algae species among order, family, and genus from South Australia used in this study

Table1: The location sites of the different algal and sponges species collected in South Australia

Sample code	Location	Sample code	Location
C9, C12 R17	Third way from Cape Jaffa to Margaret Brock Reef; near Kingston; S.A.	P1 R8, R11	Nore Creina Beach near Robe; South-East S.A.
D15, D19, D26 P2 R4, R5	Marion Reef off Edithburgh; Southern. Yorke Peninsula; S.A.	D27, D41	Kingston Jetty, far end; Kingston; SE S.A.
C2, C3	Jetty Piles; Cape Jaffa; near Kingston; S.A.	D5 P9, P11 R18, R19	D'estree's Bay; Kangaroo Island; South Australia
C11 D24 R1	Horseshoe Reef-0.65 miles; 355 degrees to Margaret Brock Light; near Kingston; S.A.	C6 D2, D6, D11, D32, D35, D36	American River; Green side of channel west of Strawbridge Point; Kangaroo Island; S.A
C5, C13	Coobowie Bay; Southern. Yorke Peninsula; S.A.	D1, D17, D29, D33, D42	Smith Bay; West of Cape D'estaing; Kangaroo Island; S.A.
C7, C10 D16, D31 P12	Margaret Brock Reef just near lighthouse (ne); near Kingston; S.A.	D7, D12, D13, D23 R6	Between Knob Point and Cape Cassini; Kangaroo Island; S.A.
C8	Cape Thomas; half way between Kingston and Robe; S.A.	D10, D21, D37 P7 R16	Point Ellen, Vivonne Bay; Kangaroo Island; S.A.
R12, R13	Port Mcdonnell Breakwater; Southern; S.A.	D3, D25	Cape D'estaing; North of Reef; Emu Bay; Kangaroo Island; S.A.
P13 R2, R3	Old Jetty Piles; Kingston Jetty; Kingston; SE; S.A.	D18, D20, D22	Smith Point; West of D'estaing; Kangaroo Island; S.A.
D40 P5, P15, P16 R10, R14, R15, R20	Godfrey Island; between Kingston and Robe; STHN. S.A.	D30, D34, D43	West of Cape D'estaing; Emu Bay; Kangaroo Island; S.A.
P3	Beachport Jetty; Beachport; S.E; S.A.	C4 P6, P8 R7, R9	Pandalowie Bay; South of lookouts; STHN Yorke Peninsula; S.A.
D14, D28, D39	1KM off Margaret Brock Lighthouse; Cape Jaffa; near Kingston; S.A.	P4, P10	Edithburgh Jetty, North of Jetty; Yorke Peninsula, S.A.
D9	Horseshoe Reef 3km wnw of Margaret Brock Lighthouse; Cape Jaffa; Kingston; S.A.	D8	Edge of Marine Reserve - Pelican Lagoon; American River; Kangaroo Island; S.A.
D38	Outside Port Mcdonnell - Deep Creek (old dairy factory creek); under bridge; S.A.	C1 P14	Point Turton Jetty; STHN. Yorke Peninsula; S.A.

D4	PORT GILES JETTY; Southern, YORKE PENIN.;S.A.		

2.2- Extract preparation

A small subsample was removed and placed in a glass vial. The sub-samples were freeze dried. After the samples were dried they were steeped in either ethanol or methanol then stored at -20°C. The extracts were frequently refreshed with either ethanol or methanol.

2.3- Sample Preparation for the Queensland Compound Library (QCL) /Compounds Australia

The extract collection was removed from the freezer one day prior to use and refreshed with methanol to the top of the vial and well mixed. A 975 µL aliquot was removed from the extract vial and pipetted into a 96 deep well Macrotitre plate. The solvent was removed under vacuum in a Savant Drier. An adhesive foil lid was placed on the samples and they were stored at -20°C until delivered to QCL/Compounds Australia on dry ice. QCL/Compounds Australia reconstituted the samples in DMSO to a final concentration of 5 mg/mL.

2.4- Preparation of sponge and macroalgae extracts samples for biological assays

Samples received from QCL were prepared in three different concentrations (0.05, 0.5 and 5 mg/ml) by dissolving the AIMS sample in DMSO. All samples were kept cold in the freezer at -20 °C.

2.5- Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 97.5%) was purchased from Sigma-Aldrich (USA). The MTT was dissolved in 1×phosphate

buffered saline (PBS) at 5 mg/ml, then filtered through 0.22 μm sterile filter (Sartorius Stedim Biotech, France) prior to use and then was stored in the freezer at -20°C. RPMI-1640 medium was purchased from Sigma-Aldrich (USA). Foetal bovine serum (FBS) was purchased from Bovogen Biologicals (East Keilor, VIC, Australia). Penicillin/streptomycin and 10× trypsin EDTA were obtained from Thermo Fisher Scientific (Scoresby, VIC, Australia). Human amyloid-β_{1-42} protein ($A\beta_{1-42}$) was obtained from Mimotopes (Melbourne, VIC, Australia). Pierce™ LDH cytotoxicity assay kit (Catalogue number: 88953) was purchased from Thermo Fisher Scientific (USA).

2.6- Cell lines and cell culture

Ordway PC-12 cells were grown in RPMI-1640 media with 10% Foetal Bovine Serum and 50 μg/ml penicillin and streptomycin. These cells were maintained in an incubator at 37 °C temperature and 5% CO_2.

PC-12 cells were treated with different concentrations of marine extract samples (0.25, 2.5, and 25 μg/mL) for 48 hours. The control group was treated with 1×PBS for the same amount of time. In addition, a solvent control contains 0.5% of DMSO in media was used to make sure that DMSO was not causing cell death. All marine extract samples were dissolved in DMSO with the final concentration less than 0.5% (v/v). All experiments were carried out in triplicate.

2.7- Cell viability assessment using MTT assay

The MTT assay is widely used to measure mitochondrial activity as an indicator of cell viability. PC-12 cells were seeded at 2×10^4 cells per well in 100 μl media in 96-well plates. The plates were kept in the incubator to attach the cells overnight. On the second day, the marine extracts were applied to the cells at final concentrations of 0.25, 2.5, and 25 μg/mL. After the treatment, the plates were incubated for 48 hours.

On the fourth day, the plate was taken out and the media removed before adding 100 µl of MTT solution at a concentration of 0.5 mg/ml in 1×PBS. The plate was kept in aluminium foil and incubated for further 2 hours at 37 °C. Then, the MTT mixture was removed from the wells and 100µl of DMSO (Sigma Aldrich) was added to each well to dissolve formazan into a coloured product that can be measured by a plate reader. The plate was read in a microplate reader (µQuant) at 570 nm. The cytotoxicity of marine extracts calculated by comparing the absorbance to cells which were treated with PBS (these cells were considered as 100% cell viability).

2.8- Preparing Aβ for treating cells

DMSO at 1% was used to dissolve non-fibrillar $A\beta_{1-42}$ to yield a protein concentration of 3.8 mM. After that, sterile PBS was added to prepare a final concentration of 100 µM. Amyloid was then dispensed into aliquots and immediately frozen at −70 °C until required.

2.9- Neuroprotection study

To assess the neuroprotective activity, only extracts that showed more than 90% cell viability at three different concentration tested earlier were selected for this assay, as treating cells with 1 µM $A\beta_{1-42}$ causing 20-30% of cell death.

PC-12 cells were used to evaluate the potential neuroprotective effects of the extracts using the MTT assay. Cells were plated at 2×10^4 cells per well in RPMI-1640 and 10% fetal bovine serum. Cells were then treated with the extracts at two different concentrations (0.25 and 25 µg/ml), either alone or 15 minutes prior to the addition of 1 µM $A\beta_{1-42}$. All cells were incubated for 48 hours. MTT was then added (0.5 mg/ml for 2 hours) and cells were lysed with DMSO. Spectrophotometric absorbance at 570 nm was then measured to assess cell viability.

2.10- LDH assay

The assay was conducted according to the manufacturer's guideline. After treating cells with extracts for 48 hours, 50 µl of media was transferred to a new plate and mixed with 50 µl of substrate and incubated in dark at room temperature for 30 minutes. After that, 50 µl of stop solution was added and the plate was read in a plate reader at 490 nm and 680 nm. The % of cytotoxicity was calculated using this formula:

$$Cell\ viability\% =$$

$$100 - [(Extract\ treated\ LDH\ activity - Spontaneous\ LDH\ activity) \div$$

$$(Maximum\ LDH\ activity - Spontaneous\ LDH\ activity) \times 100]$$

Where spontaneous LDH activity was the media control (untreated wells), while maximum LDH activity was the lysis buffer treatment that was added to the cells for 45 minutes.

2.11- Statistical analysis

All results were based on at least three independent experiments (n = 3). The effects of marine extracts on the viability of PC12 cells were analysed using one way ANOVA, followed by Tukey's honestly significant difference (HSD) post-hoc test for equal and unequal variances as appropriate. All data were analysed using SPSS software (Version 22). Differences were considered statistically significant when *p*-values were less than 0.05. Bonferroni correction was used to highlight the significant neuroprotective extracts from each sample group ($P<0.0029$ for sponge), ($P<0.007$ for green algae), ($P<0.0045$ for brown algae), and ($P<0.005$ for red algae).

3- Results

3.1 Cytotoxicity of extracts of marine sponges and macroalgae from South Australia

The cytotoxicity of 93 marine extract samples were screened against PC-12 cells using the MTT assay at three different extract concentrations (0.25, 2.5, and 25 µg/ml). The results were analysed to determine which extracts did not kill more than 10% of cells in any concentration. Tables 2-5 shows the cytotoxicity screening results after treating with marine sponge extracts, green algae, brown algae and red algae, respectively. Twenty six out of 43 sponge extracts, six out of 13 green algae extracts, five out of 16 brown algae extracts, and ten out of 20 red algae extracts showed cytotoxicity, with cell viability being less than 90% at one of the three concentrations tested (Tables 2-5). Overall, there was nearly 50% of all extracts exhibited cytotoxicity at one of the three concentrations tested, with marine sponges the most toxic (60.5% of extracts) and brown algae the least toxic extracts (only 31.25% of extracts) (Table 4). Based on the taxonomic evaluation of sponges' species (Figure 1), all the species of Ircinia genus were toxic with the exception of D8. Two third of the species from Dictyoceratida and Haplosclerida orders were toxic. Six extracts out of 13 did not show cytotoxicity in Poecilosclerida order, with all of the extracts being cytotoxic in Chondropsidae, Coelosphaeridae, and Desmacididae families. In green algae (Figure 2), all the extracts from Codiaceae family were safe to PC-12 cells. On the other hand, Caulerpaceae family was the most toxic family, as all of the species showed cell viability below 90% with the exception of C3. The taxonomic distribution of brown algae (Figure 3) showed that all the species of Sporochnales and Sphacelariales were not toxic to the cells, while almost one third of Fucales order species showing cytotoxic to PC12 cells. The taxonomic evaluation of red algae

(Figure 4) showed that both Gigartinales (with the exception of R12) and Gracilariales orders were cytotoxic, while all the species of Ceramiales order were not cytotoxic to PC-12 cells with the exception of R5.

Table 2: Summary of sponge extract toxicity against neuronal PC-12 cells at three different extract concentrations (n=3) (* p <0.05, ** p <0.01, and ***p <0.005 versus control PBS)

Sample No	Cell viability at different extract concentrations (%)			Sample No	Cell viability at different extract concentrations (%)		
	0.25 µg/mL	2.5 µg/mL	25 µg/mL		0.25 µg/mL	2.5 µg/mL	25 µg/mL
D1	78.7 ± 1.2 ***	78.7 ± 3.5 ***	75.2 ± 2.6 ***	D23	89.2 ± 1.7 *	96.5 ± 2.4	87.8 ± 2.1 *
D2	99.2 ± 2.1	96.6 ± 1.4	90.5 ± 5.1	D24	93.1 ± 3.5	92 ± 2.1	86.2 ± 2.6 *
D3	90.9 ± 3.6	90.7 ± 2.5	94.6 ± 1.1	D25	95.6 ± 1.3	93.3 ± 0.5	84.7 ± 2.9***
D4	92 ± 3.6	76.8 ± 9 *	84 ± 1.2	D26	81.1 ± 6.3 *	87.3 ± 2.2	80.9 ± 1.7 *
D5	99.8 ± 3.6	92.4 ± 1.6	88.1 ± 3.5	D27	79.5 ± 5.2 **	79.9 ± 1.8 **	80.2 ± 0.3 **
D6	81.9 ± 2.3 **	76 ± 3.3 ***	76.8 ± 2.6 ***	D28	88.5 ± 2.3 *	87.4 ± 2.6*	89.9 ± 0.5*
D7	93.3 ± 6.9	95.1 ± 1.5	77.3 ± 3.6 *	D29	80.7 ± 1.1 **	78.9 ± 4.9***	82.8 ± 1.2 *
D8	90.3 ± 2.6	90.2 ± 3.3	93 ± 4.4	D30	90.4 ± 0.4	90.9 ± 3.5	90.5 ± 2.3
D9	75.1 ± 1.7 ***	74.6 ± 1.1 ***	68.8 ± 2 ***	D31	97.3 ± 1.6	99..2 ± 0.2	96.1 ± 1.6
D10	95.7 ± 0.9	100.9 ± 3.1	94.4 ± 2.6	D32	96 ± 5.3	93.3 ± 1.4	82.4 ± 6.6
D11	96.4 ± 1.7	96.1 ± 0.5	94.2 ± 0.8	D33	95.7 ± 2.6	92.9 ± 2.5	91.3 ± 0.9
D12	84.6 ± 3.7 *	84.6 ± 2.2 *	78.5 ± 1.6 ***	D34	89.9 ± 2.4	89.7 ± 1.2	92.2 ± 3.2

D13	92.5 ± 3.6	85.8 ± 2.4	87.4 ± 1.9	D35	98.5 ± 1.8	100.6 ± 2.9	91.7 ± 0.8 *
D14	71.9± 8 **	74 ± 1.3 *	70.7 ± 1 **	D36	93.2 ± 3.9	90.3 ± 1.9	93.2 ± 0.3
D15	83.5 ± 1.9 ***	82.9 ± 1.4 ***	86.7 ± 1.3 **	D37	88.1 ± 0.9 *	89.1 ± 1.4 *	87.9 ± 2.8 *
D16	95.4 ± 2.1	92.5 ± 4.8	94.8 ± 3.6	D38	75.9 ±1.5 *	71.6± 5.4 **	74.6 ± 0.9 **
D17	92.9 ± 1.3	95.7 ± 2.6	29.9 ± 1.3 ***	D39	101.7 ± 2.2	100.6 ± 2.3	96.3 ± 0.7
D18	99.4 ± 3.8	99.6 ± 2.8	39.4 ± 2.5	D40	105.8 ± 0.8	95.2 ± 2.8	95.4 ± 3.2
D19	96.4 ± 2	90.8 ± 2.1	96.3 ± 2.7	D41	91.5 ± 1.8	87.4 ± 2.9 *	87.6 ± 1.1*
D20	80.2 ± 2.9 ***	80.7 ± 2.6 ***	80.3 ± 1.5 ***	D42	96.9 ± 3.5	94.3 ± 3.2	102.2 ± 2.5
D21	95.8 ± 4.8	96.5 ± 2.3	94.2 ± 2.5	D43	95.6 ± 1	100.1 ± 2.3	97.6 ± 0.2
D22	84 ± 0.8 ***	72.1 ± 3.3 ***	7.7 ± 0.5 ***				

Table 3: Summary of green algae extract toxicity against neuronal PC-12 cells at three different extract concentrations (n=3) (* p <0.05, ** p <0.01, and ***p <0.005 versus control PBS)

Sample No	**Cell viability at different extract concentrations (%)**			**Sample No**	**Cell viability at different extract concentrations (%)**		
	0.25 µg/mL	**2.5 µg/mL**	**25 µg/mL**		**0.25 µg/mL**	**2.5 µg/mL**	**25 µg/mL**
C1	83.9 ± 1.2 **	87.5 ± 2.1 *	80.4 ± 2.8 ***	C8	94.9 ± 1.3	92.4 ± 2.9	92.2 ± 2.3
C2	86.6 ± 4.8	82.5 ± 8.9	80.3 ± 5.5	C9	90 ± 2.7	91.9 ± 2.7	91.2 ± 2.2
C3	101.2 ± 0.2	98.9 ± 2.1	100.6 ± 0.9	C10	90.3 ± 1.4	91.3 ± 2.1	90.4 ± 2.9
C4	87.2 ± 1.3 ***	87.4 ± 1.1 ***	85.9 ± 1 ***	C11	96.9 ± 3.2	92.9 ± 1.4	94.7 ± 2.3

C5	80.9 ± 2.2 ***	81.3 ± 2 ***	78.3 ± 1.4 ***	C12	99.9 ± 2.3	98.7 ± 0.6	91.1 ± 3.2
C6	82.8 ± 2.9 ***	79.6 ± 0.2 ***	72.9 ± 1 ***	C13	91.3 ± 3.1	88.5 ± 2.3	85.9 ± 2.2 *
C7	101.9 ± 3.9	98.6 ± 4.6	98.7 ± 0.4				

Table 4: Summary of brown algae extract toxicity against neuronal PC-12 cells at three different extract concentrations (n=3) (* p <0.05, ** p <0.01, and ***p <0.005 versus control PBS)

Sample No	**Cell viability at different extract concentrations (%)**			**Sample No**	**Cell viability at different extract concentrations (%)**		
	0.25 µg/mL	**2.5 µg/mL**	**25 µg/mL**		**0.25 µg/mL**	**2.5 µg/mL**	**25 µg/mL**
P1	88.9 ± 0.7	86.5 ± 4.8	84.2 ± 3.9 *	P9	95.7 ± 3.6	92.5 ± 2.7	90.4 ± 1.7
P2	102.1 ± 0.6	104.2 ± 2	101.2 ± 1.8	P10	96.2 ± 0.8	91.9 ± 2.2	98.9 ± 2.1
P3	92 ± 2.7	92.4 ± 2.3	92.1 ± 2.1	P11	88 ± 2.8 *	83.6 ± 0.5***	81.7 ± 2.1***
P4	85.3 ± 2.9 **	89.4 ± 1.3 *	84.4 ± 1.6 **	P12	88.9 ± 3.2	91.8 ± 1.7	87.9 ± 2.1 *
P5	97.3 ± 2	93.5 ± 3	92.1 ± 1.9	P13	97.7 ± 2.7	92.5 ± 2.6	98.1 ± 2.4
P6	91.3 ± 1.9	94.2 ± 2.7	92.8 ± 3.7	P14	91.8 ± 0.9 *	90.4 ± 1.3 *	91.9 ± 1.3 *
P7	97.4 ± 1.5	96.9 ± 1.1	108.1 ± 2.5	P15	98.9 ± 1.7	95.4 ± 2	92.8 ± 1.7
P8	87.3 ± 1.5 *	87.9 ± 1.6 *	82.9 ± 2.1 ***	P16	90.9 ± 0.8 *	96.3 ± 2.2	92.7 ± 1.5

Table 5: Summary of red algae extract toxicity against neuronal PC-12 cells at three different extract concentrations (n=3) (* p <0.05, ** p <0.01, and ***p <0.005 versus control PBS)

Sample No	Cell viability at different extract concentrations (%)			Sample No	Cell viability at different extract concentrations (%)		
	0.25 µg/mL	2.5 µg/mL	25 µg/mL		0.25 µg/mL	2.5 µg/mL	25 µg/mL
R1	94.4 ± 3.3	95.2 ± 2.7	97.9 ± 1.3	R11	93.6 ± 1.5	94.3 ± 2.7	90 ± 1.3 *
R2	90.7 ± 0.3 *	90.3 ± 0.5 *	90.3± 1.3 *	R12	94.5 ± 0.5	92.6 ± 2.2	92.3 ± 0.7
R3	94.9 ± 0.9	94.3 ± 1.6	97.5 ± 2.4	R13	87.4 ± 0.8*	84.9 ± 1.4 **	82.9 ± 2.9***
R4	90.3 ± 5.9	100.6 ± 2.3	90.4 ± 1.3	R14	98.2 ± 1.4	98.7 ± 4.6	85.9 ± 2.8*
R5	86.2 ± 0.6 ***	87.7 ± 0.6 **	86.4 ± 2 ***	R15	77.9 ± 0.7***	82.5 ± 1.6 **	83.5 ± 4.2 **
R6	77.4 ± 0.5 ***	78.9 ± 1.1 ***	76.2 ± 0.9 ***	R16	86.2 ± 0.6 *	87.8 ± 3.7	88.6 ± 3.2
R7	91.7 ± 3.2	91.8 ± 1.3	96 ± 1.4	R17	89.6 ± 3.3	88.5 ± 1.5 *	88.9 ± 2.1
R8	93.9 ± 1.4	96.5 ± 2.8	92.6 ± 3.3	R18	75.4 ± 0.5***	74.1 ± 0.4***	81 ± 1***
R9	89.6 ± 1.1 *	85 ± 1.9 ***	86.6 ± 1.4 **	R19	85.1 ± 2.2 *	85.9 ± 0.3 *	83.6 ± 3.3 **
R10	94.6 ± 2	93 ± 1.7	95.1 ± 2.7	R20	99 ± 2.9	97.6 ± 3.6	99.9 ± 5.6

3.2Neuroprotective activity of extracts of marine sponges and macroalgae from South Australia

Only these extracts at any of these three concentrations showing cell viability of more than 90% were used in this assay. The sponge and macroalgae extracts were applied to PC-12 cells 15 minutes before adding $A\beta_{1-42}$ (1 µM) and the cells were incubated

for 48 hours before assessing cell viability. Treating the cells with $A\beta_{1-42}$ alone resulted in 25-35% reduction in cell viability (Figure 5-8; $P<0.05$). Sponge extracts demonstrated a varied degree of cell protection from the toxicity induced by $A\beta_{1-42}$ (Figure 5). 76% of the sponge extracts (13) showed neuroprotection at the highest concentration (25 µg/ml). More than half of the green algal and brown algal extracts significantly reduced the toxicity of $A\beta_{1-42}$ (Figure 6 and Figure 7), while only half of the red algae extracts showed neuroprotective activity (Figure 8). Interestingly, five extracts (D8, D16, D19, D21, and D40) from marine sponges showed neuroprotective activities in both tested concentrations, while ten extracts from algae [2 from green algae (C9 and C10), 4 from brown algae (P2, P3, P9, and P10), and 4 from red algae (R1, R3, R4, and R11)] were active in protecting the cells from the toxicity of Aβ in both tested concentrations. Based on the taxonomic distribution of sponges' species, extracts from all nontoxic species of Dendroceratida, Dictyoceratida, and Haplosclerida orders were neuroprotective to cells against the toxicity induced by of $A\beta_{1-42}$. Three species of the Haplosclerida order were active in both concentrations, which highlighted the potential of these orders for providing neuroprotective compounds. In green algae, extracts from half of nontoxic species of Codiaceae family showed significant neuroprotective activity. In brown algae, both extracts from Dictyopteris genus showed significant neuroprotective activities in both tested concentrations. 80% of nontoxic extracts from Fucales order were shown to protect PC-12 cells against $A\beta_{1-42}$, while all extracts from Sporochnales order failed to provide any protection. In red algae, 75% of nontoxic extracts from Ceramiales order showed neuroprotective activities, while extracts from half of species from Corallinales order were neuroprotective. Gigartinales order did not provide any neuroprotective extracts.

To confirm the significant effects of these active extracts, Bonferroni correction was used and only seven extracts (D8 and D40 from sponges; C10 from green algae; and P1, P9, P10, and P14 from brown algae) demonstrated significant neuroprotective activities. These extracts are the priorities for further studies in identifying active compounds.

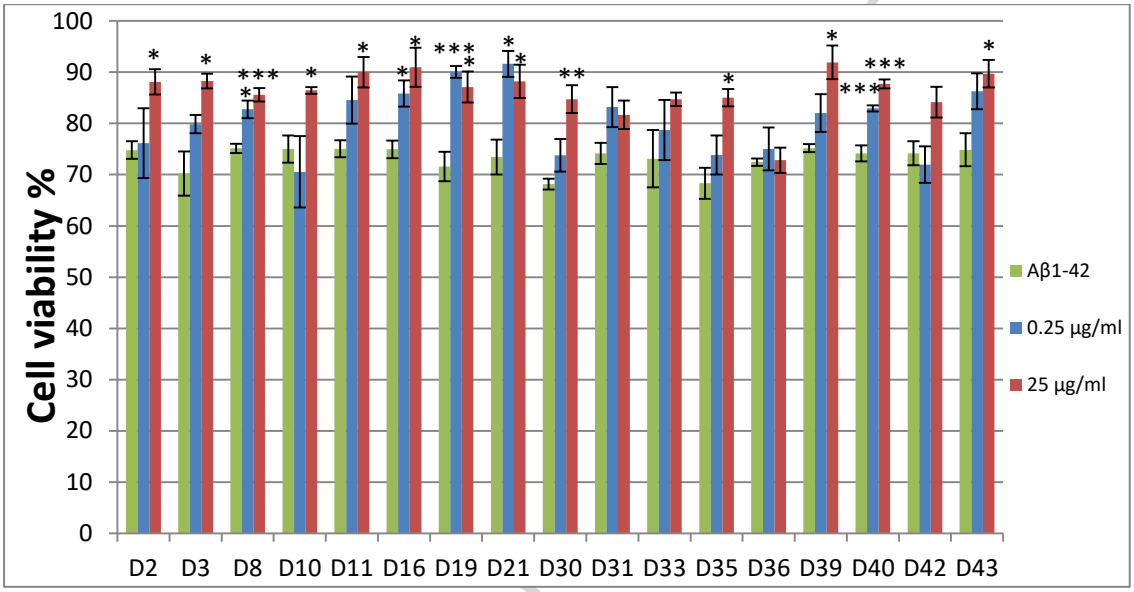

Figure 5: Neuroprotection activity of sponge extracts against $A\beta_{1-42}$ induced cytotoxicity in PC-12 cells using MTT assay showing relative cell viability (%). Each value is the mean ± SEM of three independent experiments (* p <0.05, ** p <0.01, and ***p <0.005 versus control Aβ)

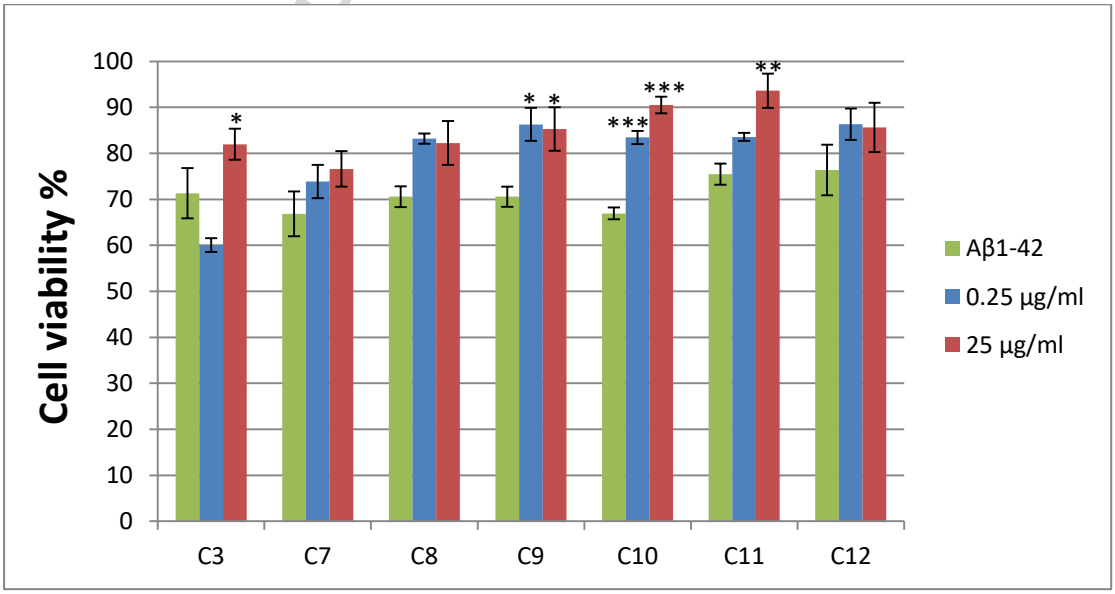

Figure 6: Neuroprotection activity of green algae extracts against $A\beta_{1-42}$ induced cytotoxicity in PC-12 cells using MTT assay showing relative cell viability (%). Each value is the mean ± SEM of three independent experiments (* p <0.05, ** p <0.01, and ***p <0.005 versus control Aβ)

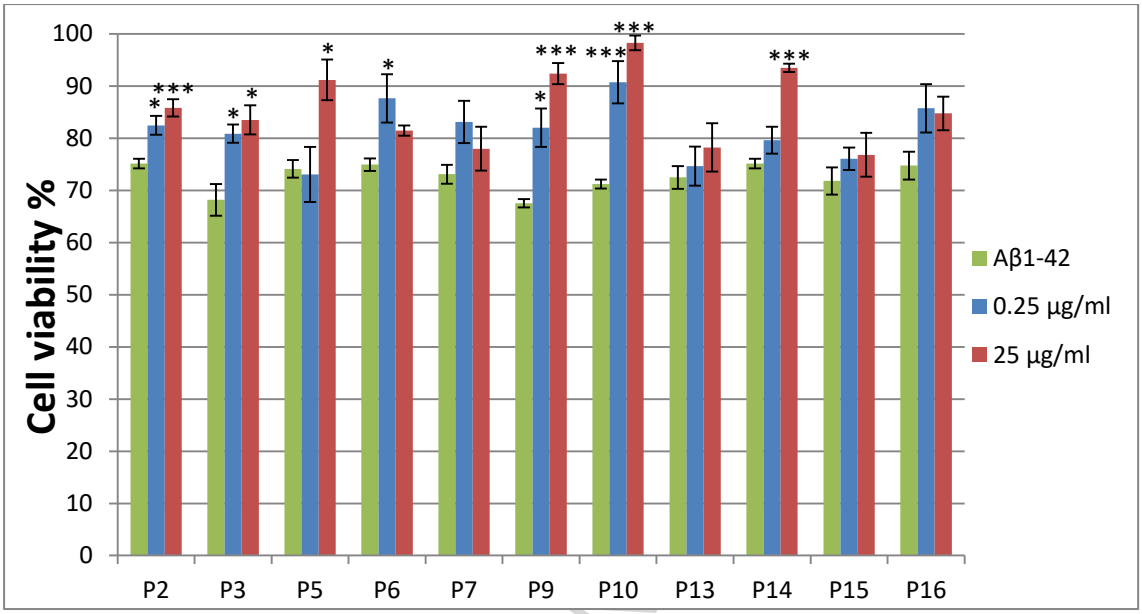

Figure 7: Neuroprotection activity of brown algae extracts against $A\beta_{1-42}$ induced cytotoxicity in PC-12 cells using MTT assay showing relative cell viability (%). Each value is the mean ± SEM of three independent experiments (* p <0.05, ** p <0.01, and ***p <0.005 versus control Aβ)

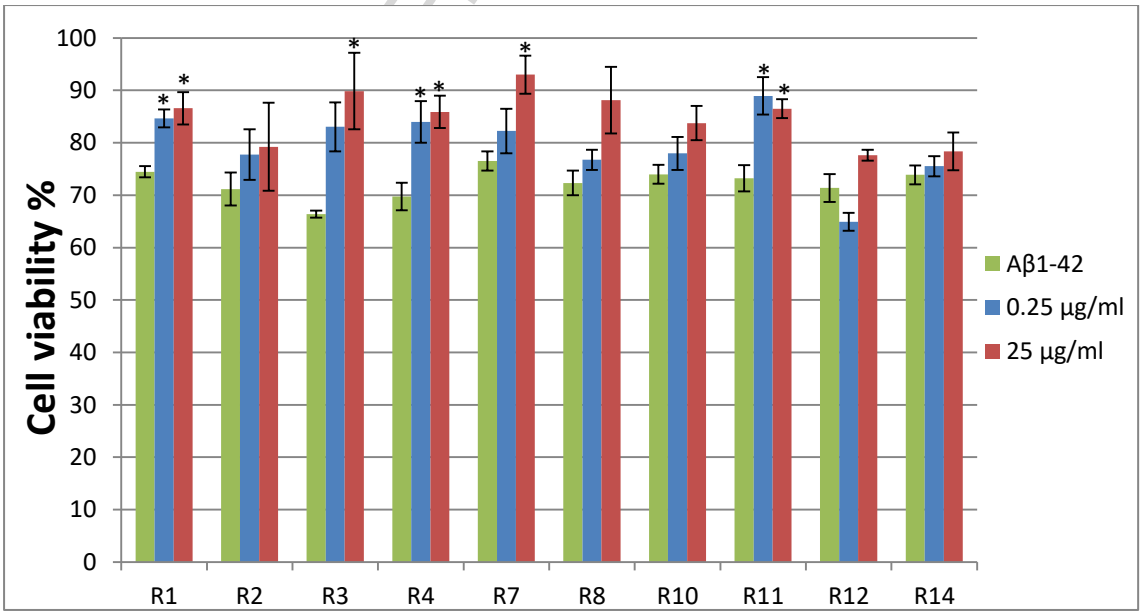

Figure 8: Neuroprotection activity of red algae extracts against $A\beta_{1-42}$ induced cytotoxicity in PC-12 cells using MTT assay showing relative cell viability (%). Each value is the mean ± SEM of three independent experiments (* p <0.05, ** p <0.01, and ***p <0.005 versus control Aβ)

3.3 Validation of cytotoxicity of selected extracts by LDH assay

To validate the cytotoxicity of extracts identified by MTT assay based on mitochondrial metabolic activity, a second assay-LDH assay was conducted to test five selected extracts (at 25 µg/mL) with the most significant neuroprotective activities. All the five selected extracts showed less than 10% of cytotoxicity as measured by LDH assay (Figure 9), similar to the results by MTT assay.

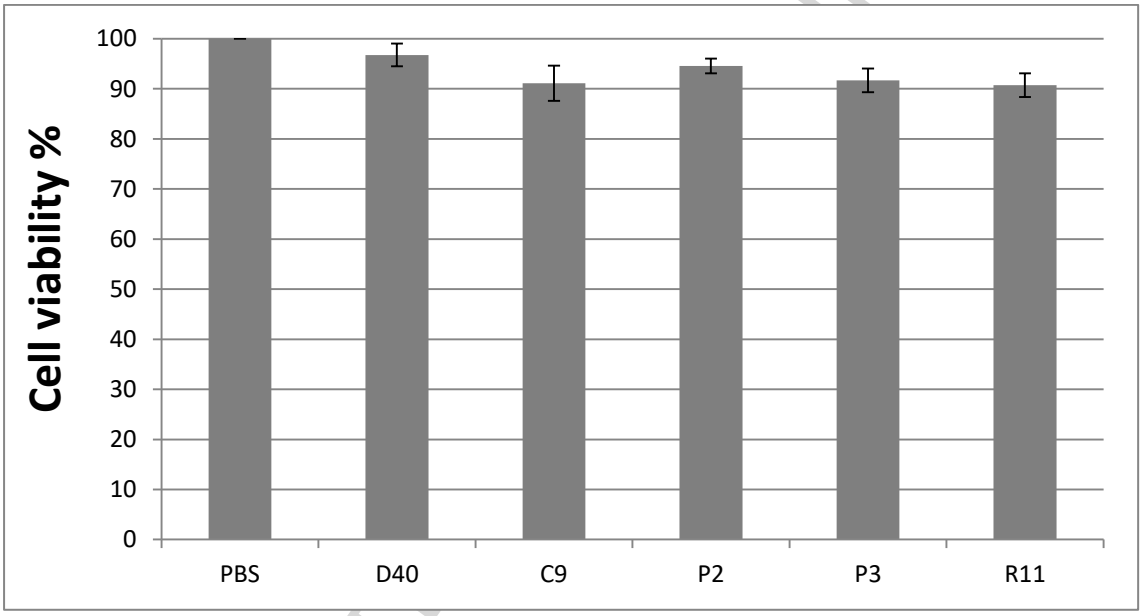

Figure 9: Relative cell viability (%) of PC12 cells estimated by LDH assay following 48 h treatment with selected extracts at 25 µg/mL. Each value is the mean ± SEM of four independent experiments

4- Discussion

60% of the marine sponge extracts tested (26 out of 43 extracts) at a concentration range of 0.25-25µg/ml were cytotoxic to PC-12 cells. Cytotoxic compounds in sponges are common given that half of the anticancer compounds discovered during 2001 to 2010 were isolated from sponges (Mehbub et al., 2014). In addition, a study showed that four dictyodendrins isolated from *Ianthella* sp. were shown to be active in reducing the activity of beta-site amyloid precursor protein cleaving enzyme

(BACE); however three of them were cytotoxic to SW620 cells (Zhang et al., 2012b). Three extracts (D17, D18, and D22) belonging to the Haplosclerida order showed the highest cytotoxicity, with cell viability less than 40%.

It is significant though that 76% (13 out of 17) of those non-toxic sponge extracts demonstrated promising neuroprotective activity, by reducing the cytotoxicity induced by Aβ.

For all the three algae classes combined, 43% of the algae extracts (21 out of 49 algae extracts) were shown to reduce cell viability to less than 90%. The algae extracts are less toxic when compared with the sponge extracts. Among these three algae classes, brown algae extracts showed the least toxic, with only 31%, when compared with 46% for green algae and 50% for red algae.

In contrast to the cytotoxicity trend, the highest percentage (44%) of brown algae extracts demonstrated neuroprotective activity against Aβ-induced cytotoxicity to PC-12 cells, followed by 31% green algae and 25% red algae.

MTT assay is based on the measurement of mitochondria metabolic activity (Mosmann, 1983), and a second assay is preferred to validate the cytotoxicity. LDH assay based on detecting lactate dehydrogenase released into media (Korzeniewski and Callewaert, 1983) was therefore used on five randomly selected five active extracts. The results from two assays are very similar and comparable, which demonstrated the reliability of the screening tests.

Based on the location sites, extracts with demonstrated neuroprotective activities were collected from 19 location sites. Two location sites were shown to have three

neuroprotective extracts, which was American River with three sponge extracts (D2, D11, and D35) and Marion Reef with 1 sponge and 2 macroalgae extracts (D19, P2, R4). In addition, six locations which include Point Ellen (D10 and D20), Margrete Brock (D16, C10), West of Cape (D30 and D43), Godfrey Island (D40 and P5), Horseshoe Reef-0.65 miles (C11 and R1), and Pandalowie Bay (P6 and R7) showed to have 2 extracts with neuroprotective activities. This highlights the diverse activities among different location sites.

Based on sponge taxonomy, all of the orders in this study with the exception Biemnida order have been reported before to have compounds with neuroprotective activities. In addition, 7 families (Dysideidae, Ircinidae, Thorectidae, Niphatidae, Desmacididae, Halichondriidae, and Aplysinidae) have been reported before to have neuroprotective compounds (Alghazwi et al., 2016a).

Table 6: Summary of sponge, green algae, brown algae, and red algae showing cytotoxicity and neuroprotective activity against PC-12 cells

Marine organism	Total screened extracts	Total toxic extracts	Total neuroprotective extracts by one way ANOVA	Total neuroprotective extracts by Bonferroni correction
Sponge	**43**	**26 (60%)**	**13 (30%)**	**2 (4.7%)**
Green algae	**13**	**6 (46%)**	**4 (30.7%)**	**1 (7.7%)**
Brown algae	**16**	**5 (31.25%)**	**7 (43.75%)**	**4 (25%)**
Red algae	**20**	**10 (50%)**	**5 (25%)**	**0 (0%)**
Total	**92**	**47 (51.1%)**	**29 (31.5%)**	**7 (7.6%)**

Even though many sponges and macroalgae extracts were reported to show neuroprotective activity previously from similar families to the one used in our studies, many unreported families in this study were found to possess neuroprotective

activity for the first time. From sponge samples, only four of these extracts were reported previously from a similar family, and nine of these extracts were reported for the first time. Interestingly, all of the green algae extracts were reported before by other researchers from a similar family to show neuroprotective activity. In red algae, 2 families were reported previously, and 3 families were yet to be reported (Table 7). All brown algal extracts are reported here to show neuroprotective activity for the first time. This highlights the diverse species in South Australia, and also their great potential for neuroprotective drug discovery, as all the species are reported for the first time.

In contrast, lack of neuroprotective activity or cytotoxicity was presented in some extracts from similar family or genus to the reported neuroprotective compounds in the literature. This include *Cribrochalina* genus, as extract D23 was toxic and excluded from further studies. Two compounds, namely cribronic acid (2S,4R,5R)-5-hydroxy-4- sulfooxypiperidine-2-carboxylic acid and (2S,4S)-4-sulfooxypiperidine-2-carboxylic acid, isolated from *Cribrochalina olemda* have reported to have glutamate receptor inhibition activity (Sakai et al., 2003). Hymenialdisine isolated from *Halichondriidae* demonstrated kinase inhibition by reducing the activity of GSK- 3β and CK1 (Meijer et al., 2000). However, in our screening, extract D14 isolated from *Halichondriidae* was toxic, with observed cell viability of less than 80%. Three compounds; spiralisone A, spiralisone B and chromone 6 were isolated from *Zonaria spiralis* and found to inhibit kinase activity (Zhang et al., 2012d). Extract P3 is from the same genus *Zonaria* and was found to be toxic to PC-12 cells. Many compounds isolated from *Sargassum* genus demonstrated neuroprotective activities. Compounds such as (5E,10Z)-6,10,14-Trimethylpentadeca-5,10-dien-2,12-dione and (5E,9E,13E)-6,10,4-trimethylpentadeca-5,9,13-trien-2,12-dione were previously shown to reduce

cholinesterase activity (Ryu et al., 2003). Fucoxanthin demonstrated DPPH radical-scavenging activity (Yan et al., 1999) and inhibited cell toxicity induced by hydrogen peroxide (Heo et al., 2008). Three plastoquinone and nineteen meroditerpenoid compounds demonstrated antioxidant activity by reducing lipid peroxidation and radical scavenging against DPPH (Mori et al., 2005), (Jung et al., 2008). Alginic acid has shown anti-inflammatory activity (Sarithakumari and Kurup, 2013) and four compounds; MC14 (Tsang et al., 2001), sargaquinoic acid (Kamei and Tsang, 2003), sargachromenol (Tsang et al., 2005) and pheophytin A (Ina et al., 2007) have shown enhanced neuronal growth. Only one extract, P11 of *Sargassum* genus tested in our study reduced cell viability to less than 90%.

The most promising orders from sponge taxonomy are the Haplosclerida order, as both provided three neuroprotective extracts at both tested concentrations. Moreover, the Dictyoceratida order provided four neuroprotective extracts. From green algae, *Codium* genus seems to be promising as it provides three neuroprotective extracts. From brown algae, two neuroprotective extracts were isolated from *Dictyopteris* genus and three others from the Sargassaceae family. The Ceramiales order provided three neuroprotective extracts, while Corallinaceae family provided two in the red algae. For that reason, more research should be conducted in these families and orders to derive neuroprotective compounds.

Table 7: Summary of reported compounds/extracts from sponge, green algae, brown algae, and red algae from similar family or genus in this study with their reported activities

Genus/ Family	**Extract number**	**Reported compound/ extract**	**Reported activity**	**Reference**
Dysidea genus	D3	Dysiherbaine	kainic acid (KA) receptor and [3H]α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid (AMPA) receptor inhibitors	(Sakai et al., 2001b)
		Neodysiherbaine A	Inhibiting KA and AMPA receptors	(Sakai et al., 2001a)
		Dysibetaine CPa and dysibetaine CPb	Inhibiting N-methyl-D-aspartic acid-type (NMDA) and KA receptors	(Sakai et al., 2004)
		Dysideamine	Reduce reactive oxygen species (ROS) formation	(Suna et al., 2009)
Ircinia genus	D8	Methanol extracts from *Ircinia spinulosa* and *Ircinia fasciculate*	Acetylcholinesterase inhibitor activity	(Orhan et al., 2012)
		Isopropanolic extract from *Ircinia dendroides*	GSK-3β	(Bidon-Chanal et al., 2013)
Amphimedon genus	D21	Ethyl acetate extract from Amphimedon navalis	Acetylcholinesterase inhibition activity	(Beedessee et al., 2013)
Aplysina genus	D40	NP04634 from Aplysina cavernicola	Reduce to toxicity in bovine adrenal medullary chromaffin induced by calcium	(Valero et al., 2009)

Caulerpa genus	C3	Racemosin A and racemosin B	BACE inhibitors	(Liu et al., 2013)
		α-tocospirone, (23E)-3β-hydroxystigmasta-5,23-dien-28-one, and (22E)-3β-hydroxycholesta-5,22-dien-24-one	BACE inhibitors	(Yang et al., 2015)
		Caulerpin	Anti-inflammatory effects	(de Souza et al., 2009)
Codium genus	C9, C10, and C11	Clerosterol isolated from *Codium fragile*	Anti-inflammatory activity	(Lee et al., 2013)
Rhodomelaceae family	R3 and R4	3-(2,3-Dibromo-4,5-dihydroxybenzyl)pyrrolidine-2,5-dione, methyl 4-(2,3-Dibromo-4,5-dihydroxybenzylamino)-4-oxobutanoate, 4-(2,3-Dibromo-4,5-dihydroxybenzylamino)-4-oxobutanoic acid, 3-Bromo-5-hydroxy-4-methoxybenzamide, and 2-(3-Bromo-5-hydroxy-4-methoxyphenyl) acetamide	Reduce oxidative stress	(Li et al., 2012)

The extracts that have shown positive results by inhibiting Aβ toxicity in PC-12 cells are likely to work via different and possibly multiple mechanisms. These mechanisms include anti-aggregation effects against Aβ, anti-oxidant activity, or kinase inhibitory

activity. For instance, these extracts might be able to inhibit BACE which is an important enzyme in the aberrant production of Aβ from amyloid precursor protein (APP). Another possible mechanism is that the extracts are able to reduce reactive oxygen species (ROS) which occurs as a consequence of Aβ exposure (Butterfield and Boyd‐Kimball, 2004). For example, pinostrobin was found to inhibit the toxicity of Aβ via inhibiting intracellular ROS (Xian et al., 2012). Aβ can induce apoptosis as well, so these extracts might inhibit caspase 3 and 9, or inhibit Bcl and Bax. Previously, astaxanthin has been found to inhibit Aβ toxicity via the apoptosis pathway, inhibiting Bax and upregulating Bcl-2, while also upregulating HO-1 expression (Wang et al., 2010).

Further studies are required to isolate and purify the active compounds from these active extracts identified. Novel compounds are expected to be discovered by further mechanistic insight into cellular pathways for neuroprotection.

5- Conclusion

This study has demonstrated that marine sponges and macroalgae collected in South Australia are a potential source of neuroprotective compounds, with one-third of 92 extracts screened (29 extracts) found to reduce neurotoxicity induced by Aβ in PC-12 cells. More than half of these extracts (15 extracts out of 29 extracts) were active in both tested concentrations. 65% of these sponge and macroalgae extracts from genus that have not been reported before, to demonstrate protection against Aβ-neurotoxicity. These results are of significance for further studies isolating and characterizing the active compounds from these extracts and understand their mechanisms of actions for potential neuroprotective drug discovery and development.

Conflict of interest

The authors confirm that this article content has no conflict of interest.

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Highlights

- 60% of South Australian marine sponge extracts demonstrated cytotoxicity
- Brown algae demonstrated the least cytotoxicity among other algae classes
- About 31.5% of extract demonstrated neuroprotective activities